REMARKS

The Office Action of November 2, 2005 has been received and reviewed.

Claims 1-17, 19 and 20 are currently pending in the application.

Claims 1-17 stand rejected.

Claims 19 and 20 are objected to.

Claim 1 has been amended to recite the limitations recited by claim 19.

Claim 3 has been amended to remove the phrase "a part or all of" to be consistent with amended claim 1 and to further clarify the term "factor" by adding the word "protein." Claims 4 and 5 have been amended to also recite the phrase "protein factors."

Claim 6 has been amended to add the word "other" to more clearly indicate what is being claimed.

Claim 12 has been amended to recite the limitations recited by claim 20 and to further clarify the term "factor" by adding the word "protein." Claims 13-15 have been amended to also be consistent with amended claim 12.

Claim 17 has been amended to be consistent with amended claim 12.

Claims 19 and 20 have been cancelled.

All cancellations and amendments are made without prejudice or disclaimer. Reconsideration is respectfully requested.

Rejections Under 35 U.S.C. § 102(e)

Claims 1-17 stand rejected under 35 U.S.C. § 102(e) as assertedly being anticipated by Rothschild et al., U.S. Patent No. 6,306,628 (hereinafter referred to as "Rothschild et al."). (*See,*

Office Action of November 2, 2005, at page 2, hereinafter referred to as "Office Action"). Applicants traverse the rejection as set forth herein.

Summary of the Present Invention in Relation to Cited References

To date, when protein was expressed in the cell *in vivo*, the protein of interest was labeled or marked with His-tag or an equivalent in order to isolate the produced protein from the cell extract. Similarly, when the protein of interest was produced within an *in vitro* reaction system, the same labeling method was employed wherein the protein of interest was labeled as described in Appendix 1 and 2, as follows (complementary copies of references attached hereto as Exhibits A and B):

Appendix 1: *Methods in EnzymologyMethods in Enzymology*, 236:245-250 (2000)

This is an authentic text book in the field of enzymology. At the beginning of page 245, it is disclosed that the "expression and subsequent purification of recombinant proteins are widely employed in biochemical studies. A powerful purification method involves the use of peptide affinity tags, which are fused to the protein of interest and used to expedite protein purification via affinity chromatography." Furthermore, this text book describes many purification procedures of "the tagged protein of interest." Therefore, this text book teaches that the "protein of interest" should be labeled with His-tag or an equivalent to isolate or purify it from the reaction mixture.

Appendix 2: EasyXpress™ Protein Synthesis Handbook (September 2003, QIAGEN).

This QIAGEN Kit Manual for transcription/translation system discloses a kit wherein *E. coli* is used to provide the necessary components for transcription/translation reactions. But the

components are not labeled or marked. Instead, the protein of interest is labeled with His-tag and purified by Ni-NTA column chromatography. QIAGEN's kit is still used today by many scientists.

In contrast, the present invention upsets this typical methodology and approach to protein expression. The present invention discloses an *in vitro* transcription/translation method, wherein all protein components constituting the *in vitro* transcription/translation reaction system are labeled or marked with His-tag or an equivalent. This is stark contrast to the commonly employed methodologies wherein only the protein of interest is labeled. Therefore, by using methods such as affinity chromatography, all the protein components of the reaction system can simply be isolated from the produced protein after translation.

The Examiner states that claims 19 and 20 or objected to as being dependent upon rejected base claims "but would otherwise be allowable if rewritten in independent form." (*Id.* at page 6). Although Applicants do not agree that Rothschild et al. discloses every element of the present claims, to expedite prosecution, Applicants have amended independent claims 1 and 12 to recite the limitations of dependent claims 19 and 20, as suggested by the Examiner. Dependent claims thereto have been amended to be consistent with the amendments to claims 1 and 12. Claim 1 has been further amended to recite, in part, "translation of the peptide or peptide derivative to allow the separation of the produced peptide or a peptide derivative from the labeled protein components constituting the reaction system" to more clearly indicate that which Applicants intend as the subject matter of the claim. Support for this amendment may be found at, for instance, paragraph [0022] of the as-filed specification.

Furthermore, dependent claims have been amended to more clearly state that which is being claimed. Specifically, claim 3 has been amended to remove the phrase "a part or all of" to be consistent with amended claim 1, which now recites "all protein components." Claims 3-5 have also been amended to further clarify the term "factor" by adding the word "protein." Support for this amendment may be found at, for instance, paragraphs [0014], [0018] and [0042] – [0047], of the as-filed specification.

Claim 6 has been amended to add the word "other" to more clearly indicate the antecedent, "other enzymes required in the constitution of the reaction system" as recited in claim 5.

Claim 12 has been amended to recite the limitations recited by claim 20. That is, claim 12, as amended, recites, in part, "all protein components of the system which are labeled."

Claim 12 has been further amended to further clarify the term "factor" by adding the word "protein," as described for the amendments to claims 3-5. Claims 13-15 have been amended to also recite the phrase "protein factor." Support for this amendment can be found at, for instance, paragraph [0014] in the as-filed specification.

Claim 17 has been amended to be consistent with amended claim 12.

Claims 19 and 20 have been cancelled because the elements of these claims have been incorporated into independent claims 1 and 12.

Dependent claims 2-11 and 13-17 are not anticipated as, *inter alia*, depending from non-anticipated base claims, claims 1 and 12.

Reconsideration and withdrawal of the anticipation rejection of claims 1-17 are respectfully requested.

ENTRY OF AMENDMENTS

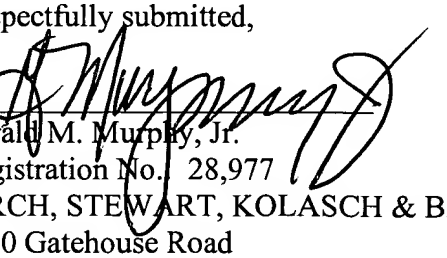
The proposed amendments to claims 1, 3-6, 12-15 and 17 should be entered by the Examiner because the amendments are supported by the as-filed specification and drawings and do not add any new matter to the application. The proposed amendments should also be entered since they comply with requirements as to form, and place the application in condition for allowance. Further, the amendments do not raise new issues or require a further search since the amendments incorporate elements from dependent claims into independent claims. Finally, if the Examiner determines that the amendments do not place the application in condition for allowance, entry is respectfully requested since they certainly remove issues for appeal.

If the Examiner has any questions or comments, please contact Thomas J. Siepmann, Ph.D., Registration No 57,374 at the offices of Birch, Stewart, Kolasch & Birch, LLP.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to our Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under § 1.17; particularly, extension of time fees.

Dated: March 2, 2006

Respectfully submitted,

By 
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Attachments: Exhibits A & B

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TJS

[16] Purification of Proteins Using Polyhistidine Affinity Tags

By JOSHUA A. BORNHORST and JOSEPH J. FALKE

The expression and subsequent purification of recombinant proteins are widely employed in biochemical studies. A powerful purification method involves the use of peptide affinity tags, which are fused to the protein of interest and used to expedite protein purification via affinity chromatography.[1,2] A widely employed method utilizes immobilized metal-affinity chromatography (IMAC) to purify recombinant proteins containing a short affinity tag consisting of polyhistidine residues. IMAC is based on the interactions between a transition metal ion (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) immobilized on a matrix and specific amino acid side chains. Histidine is the amino acid that exhibits the strongest interaction with immobilized metal ion matrices, as electron donor groups on the histidine imidazole ring readily form coordination bonds with the immobilized transition metal. Peptides containing sequences of consecutive histidine residues are efficiently retained on IMAC column matrices. Following washing of the matrix material, peptides containing polyhistidine sequences can be easily eluted by either adjusting the pH of the column buffer or adding free imidazole to the column buffer.[3]

IMAC is a versatile method that can be utilized to rapidly purify polyhistidine affinity-tagged proteins, resulting in 100-fold enrichments in a single purification step.[4] Affinity-tagged protein purities can be achieved at up to 95% purity by IMAC in high yield.[5,6] Purification using polyhistidine tags has been carried out successfully using a number of expression systems, including *Escherichia coli*,[7] *Saccharomyces cerevisiae*,[8] mammalian cells,[9]

[1] J. Nilsson, S. Ståhl, J. Lundeberg, M. Uhlén, and P. Å. Nygren, *Protein Expr. Purif.* **11**, 1 (1997).
J. W. Jarvik and C. A. Telmer, *Annu. Rev. Genet.* **32**, 601 (1998).
[3] J. Porath, *Protein Expr. Purif.* **3**, 263 (1992).
[4] J. Schmitt, H. Hess, and H. G. Stunnenberg, *Mol. Biol. Rep.* **18**, 223 (1993).
[5] E. Hochuli, W. Bannwarth, H. Döbeli, R. Gentz, and D. Stüber, *Bio/Technology* **6**, 1321 (1988).
[6] R. Janknecht, G. de Martynoff, J. Lou, R. A. Hipskind, A. Nordheim, and H. G. Stunnenberg, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8972 (1991).
[7] M. W. Van Dyke, M. Sirito, and M. Sawadogo, *Gene* **111**, 99 (1992).
[8] D. C. Kaslow and J. Shiloach, *Bio/Technology* **12**, 494 (1994).
[9] R. Janknecht and A. Nordheim, *Gene* **121**, 321 (1992).

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and baculovirus-infected insect cells.[10] However, this purification method may not be sufficient for tagged proteins expressed at low levels that require significantly greater than 100-fold enrichment or for the preparation of highly homogeneous protein samples. In such cases, either the use of a different affinity tag or the use of the polyhistidine tag in conjunction with additional purification techniques should be considered.

General Considerations

Incorporation of the Polyhistidine Affinity Tag

Affinity tags consisting of six polyhistidine residues are commonly used in IMAC. Whereas tags of six histidine residues are generally long enough to yield high-affinity interactions with the matrix, both shorter and longer affinity tags have been used successfully. In some cases the use of longer polyhistidine tags has resulted in increased purity due to the ability to use more stringent washing steps.[11] Still, it is advisable to use the smallest number of histidine residues as required for efficient purification to minimize possible perturbation of protein function. In general, a six histidine tag is an appropriate choice for the first trial when adding a novel polyhistidine tag to a protein.

Polyhistidine affinity tags are commonly placed on either the N or the C terminus of recombinant proteins. Optimal placement of the tag is protein specific. A potential problem is inaccessibility of the protein tag to the immobilized metal due to occlusion of the tag in the folded protein. Moving the affinity tag to the opposite terminus of the protein or carrying out the purification under denaturing conditions often resolves this problem. In principle, it is possible that the affinity tag may interfere with protein activity, although the relatively small size and charge of the polyhistidine affinity tag ensure that protein activity is rarely affected. Thus, the affinity tag usually does not need to be removed following protein purification.[12] If necessary, the affinity tag can be removed by use of a protease cleavage site inserted between the tag and the protein.[13]

Polyhistidine affinity tags are small enough to be incorporated easily into any expression vector. These tags can be added onto target genes by

[10] A. Kuusinen, M. Arvola, C. Oker-Blom, and K. Keinänen, *Eur. J. Biochem.* **233,** 720 (1995).

[11] R. Grisshammer and J. Tucker, *Protein Expr. Purif.* **11,** 53 (1997).

[12] J. Crowe, H. Döbeli, R. Gentz, E. Hochuli, D. Stüber, and K. Henco, *Methods Mol. Biol.* **31,** 371 (1994).

[13] D. B. Nikolov, S. H. Hu, J. Lin, A. Gasch, A. Hoffmann, M. Horikoshi, N. H. Chua, R. G. Roeder, and S. K. Burley, *Nature* **360,** 40 (1992).

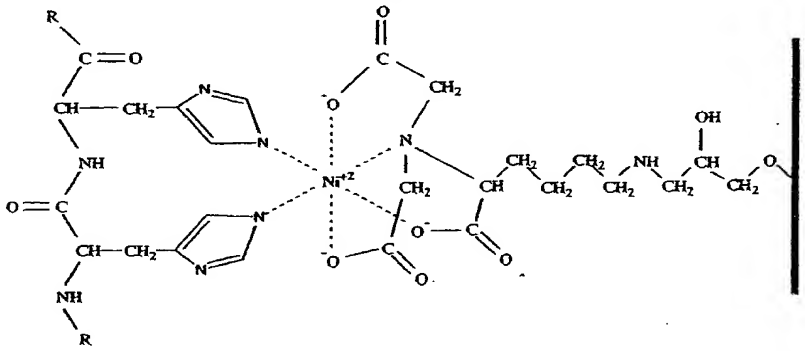
site-directed mutagenesis or by polymerase chain reaction methods. DNA fragments coding for the polyhistidine affinity tag can also be created from synthetic oligonucleotides and cloned into an appropriate location in the desired plasmid.[14] Alternatively, there are a wide variety of commercially available cloning vectors for the generation and expression of polyhistidine-tagged recombinant proteins in different expression systems. Vectors for secreted polyhistidine affinity tagged proteins have also been developed for *E. coli*.[15]

Affinity Matrices

A variety of immobilized metal matrices are available for use in IMAC. Initial IMAC reports used iminodiacetic acid (IDA) as a matrix to chelate transition metals through three coordination sites.[16] A problem with the use of IDA matrices is that the metal ion is only weakly bound to such a three-coordinate matrix. Metal leaching from the matrix during purification causes lowered yields and impure products.[12] More recently, purification of polyhistidine affinity-tagged proteins has been facilitated by the development of the commercially available matrices nickel–nitrilotriacetic acid (Ni^{2+}-NTA)[17] and Co^{2+}–carboxylmethylaspartate (Co^{2+}-CMA),[18] which are coupled to a solid support resin. These matrices securely coordinate metal ions through four coordination sites while leaving two of the transition metal coordination sites exposed to interact with histidine residues in the affinity tag. Molecular models of these interactions are shown in Fig. 1. Much of the versatility of IMAC stems from the ability of these resins to tolerate a wide range of conditions, including the presence of protein denaturants and detergents. The stability of metal binding in these resins also allows the resins to be regenerated and reused several times. The Ni^{2+}–NTA matrix (available from Qiagen) has a binding capacity of 5–10 mg protein/ml of matrix resin and a high binding affinity ($K_d = 10^{-13}$ *M*) for the six residue polyhistidine tag at pH 8.0.[4] The Co^{2+}–CMA matrix (Talon resin, available from Clontech) has a somewhat lower affinity for the polyhistidine affinity tag than the Ni^{2+}–NTA resin, resulting in elution of the tagged proteins under milder conditions. The Co^{2+}–CMA also has been reported to exhibit less nonspecific protein binding than the Ni^{2+}–NTA

[14] N. E. David, M. Gee, B. Andersen, F. Naider, J. Thorner, and R. C. Stevens, *J. Biol. Chem.* **272**, 15553 (1997).
[15] A. Skerra, *Gene* **141**, 79 (1994).
[16] J. Porath, J. Carlsson, I. Olsson, and G. Belfrage, *Nature* **258**, 598 (1975).
[17] E. Hochuli, H. Döbeli, and A. Schacher, *J. Chromatogr.* **411**, 177 (1987).
[18] G. Chaga, J. Hopp, and P. Nelson, *Biotechnol. Appl. Biochem.* **29**, 19 (1999).

a. Nickel-nitriloacetic acid (Ni^{+2}-NTA)



b. Cobalt-carboxylmethylaspartate (Co^{+2}-CMA)

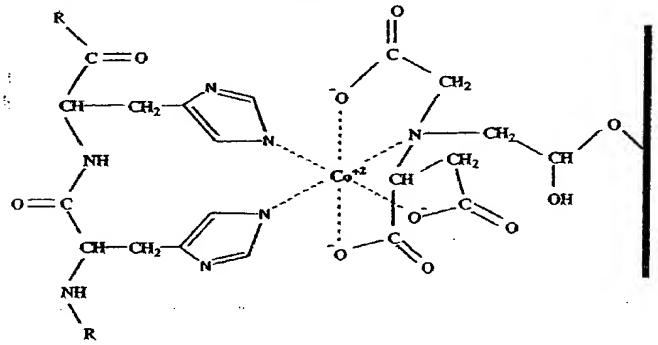


FIG. 1. Models of the interactions between the polyhistidine affinity tag and two immobilized metal affinity chromatography matrices. (a) The nickel–nitrilotriacetic acid matrix (Ni^{+2}–NTA) [from J. Crowe, H. Döbeli, R. Gentz, E. Hochuli, D. Stüber, and K. Henco, *Methods Mol. Biol.* **31**, 371 (1994)]. (b) The cobalt–carboxylmethylaspartate matrix (Co^{2+}–CMA) (from G. Tchaga, Clontech, personal communication). In both cases, the metal ion exhibits octahedral coordination by four matrix ligands and two histidine side chains, the latter provided by the polyhistidine affinity tag.

resin, resulting in higher elution product purity.[19] The binding capacity of the Co^{2+}–CMA resin is also about 5–10 mg of protein/ml of resin.

Purification under Native and Denaturing Conditions

Purification using the polyhistidine tag can be performed under either native or denaturing conditions by IMAC. The use of mild buffer conditions

[19] T.-T. Yang, P. S. Nelson, G. L. Bush, D. I. Meyer, and S. R. Kain, *Am. Biotechnol. Lab.* **I**, 12 (1997).

and imidazole as the elutant often yields biologically active purification products. Proteins that remain soluble in the cytoplasm, or that are secreted, usually can be purified using these native conditions. However, purification under native conditions may be hindered if the target protein is insoluble, aggregates in inclusion bodies, or possesses a tertiary structure that occludes the polyhistidine affinity tag. In such cases, proteins can be purified by the use of denaturing conditions such as 6 *M* guanidinium hydrochloride or 8 *M* urea during the purification process. Interaction of the resin with the polyhistidine tag does not require a specific conformation of the peptide tag, which makes effective purification with the use of denaturing conditions possible. Purification under denaturing conditions can depress the activity of phosphatases and proteolytic enzymes.[6] The use of urea as a denaturant is often preferable as 6 *M* guanidinium hydrochloride precipitates in the presence of SDS, interfering with subsequent SDS–PAGE analysis. Proteins purified under denaturing conditions can then be refolded into their active states by dialyzing away the denaturants.[20] In some cases, proteins can be refolded while bound to the resin.[21]

Purification of Membrane Proteins

Polyhistidine-tagged membrane proteins can be purified by IMAC using detergent-containing buffers to solubilize the proteins during the chromatographic process.[22,23] IMAC of membrane proteins has been carried out successfully in a variety of ionic and nonionic detergents. It is difficult to predict which detergent will be most suitable for IMAC in a given membrane protein system.[11] Although caution should be used, the Ni^{2+}–NTA and Co^{2+}–CMA matrices are generally able to tolerate limited amounts of nonionic and ionic detergents. Following IMAC, it is possible to restore the activity of purified polyhistidine-tagged membrane proteins by reconstitution into membrane vesicles.[14]

Nonspecific Binding

A problem with the use of polyhistidine affinity tags is nonspecific binding of untagged proteins. Although histidine occurs relatively infrequently (2% of all protein residues are histidine), some cellular proteins contain two or more adjacent histidine residues.[4] These proteins have an

[20] P. T. Wingfield, *in* "Current Protocols in Protein Science" (J. E. Coligan, B. M. Dunn, H. L. Ploegh, D. W. Speicher, and P. T. Wingfield, eds.), p. 6.1.1. Wiley, New York, 1995.
[21] D. Sinha, M. Bakhshi, and R. Vora, *Biotechniques* **17,** 509 (1994).
[22] R. Flachmann and W. Kühlbrandt, *Proc. Natl. Acad. Sci. U.S.A.* **93,** 14966 (1996).
[23] J. J. Janssen, P. H. Bovee-Geurts, M. Merkx, and W. J. DeGrip, *J. Biol. Chem.* **270,** 11222 (1995).

affinity for the IMAC matrix and may coelute with the protein of interest, resulting in significant contamination of the final product. This problem is generally more pronounced in systems other than *E. coli*. Mammalian systems, for example, have a higher natural abundance of proteins containing consecutive histidine residues.[12] Disulfide bond formation between the protein of interest and other proteins can also lead to contamination. The use of 10 m*M* 2-mercaptoethanol in the loading, wash, and elution buffers generally eliminates this potential problem. Nonspecific hydrophobic interactions can also cause some copurification with the desired protein. Including low levels (up to 1%) of the nonionic detergent Triton X-100 or Tween 20 in the protein buffers can reduce these interactions without substantially affecting the binding of the tagged protein to the Ni^{2+}–NTA or the Co^{2+}–CMA matrices. The addition of salt (up to 500 m*M* NaCl), glycerol (up to 20%), or low levels of ethanol (up to 20%) can also reduce nonspecific hydrophobic protein interactions with these matrices. Optimum levels of these buffer components should be determined experimentally for individual proteins.

Purification Procedure

Design of Protein Binding, Washing, and Elution Steps

Binding of the polyhistidine-tagged proteins can be performed using either a column or a batch procedure. Cell lysis should be done in buffered solution adjusted to pH 8.0. When the column procedure is utilized, the resin is packed into a column and the cell lysate is slowly loaded (3 to 4 column volumes per hour) onto the column. The batch procedure involves incubating the affinity matrix resin in the cell lysate solution and then packing the resin into a column. During incubation at 4°, the resin can be suspended in the cell lysate solution by shaking or stirring. Use of the batch procedure often results in more efficient binding of the tagged protein. With either method, the use of the minimum amount of resin needed to bind the tagged protein is recommended. The tagged protein usually has a higher binding affinity than other proteins that bind nonspecifically to the resin. Thus, when the minimum amount of resin is used, the tagged protein will fill most of the available binding sites, reducing the number of nonspecific proteins that bind. Sodium chloride (up to 500 m*M*) and low levels of imidazole (up to 20 m*M*) can also be included in the binding buffer to reduce the number of proteins that bind nonspecifically to the resin. Most protease inhibitors, with the exception of metal-chelating agents such as EDTA, can be included in all buffers. There are commercially

available protease inhibitor cocktails specifically designed for use in IMAC purifications (Sigma).

Following binding of the tagged protein, the column can be washed to remove nonspecific proteins that bind weakly to the column. If desired, the inclusion of imidazole (10–50 m*M* for Ni^{2+}–NTA, 10 m*M* for Co^{2+}–CMA) in the wash buffer will increase the stringency of the wash and elute nonspecifically bound proteins more effectively. Alternatively, a wash buffer with a pH lower than that of the binding buffer (pH 6.3 for Ni^{2+}–NTA, pH 7.0 for Co^{2+}–CMA) can be employed to remove nonspecifically bound proteins. Agents such as detergents, 2-mercaptoethanol, and sodium chloride are often included in the wash and binding buffer to reduce nonspecific protein binding.

Three different methods can be used to elute the tagged protein of interest. Lowering the pH (to 5.3–4.5 for Ni^{2+}–NTA, 6.0 for Co^{2+}–CMA) protonates the imidazole nitrogen atom of the histidine residue (pK_a 6.0) and disrupts the coordination bond between the histidine and the transition metal. The histidine analog imidazole can also be used to competitively elute the bound polyhistidine residues (concentrations of 100 m*M* or higher for Ni^{2+}–NTA, 50 m*M* or higher for Co^{2+}–CMA). If the tagged protein forms oligomers, more stringent conditions, such as lower pH or higher concentrations of imidazole, may be required to elute the protein. While both of these elution methods are effective, the use of imidazole is often preferable as exposure to low pH may damage the protein of interest. Note, however, that heating a sample that contains imidazole to boiling prior to SDS–PAGE can cause acid-labile bonds to hydrolyze. Instead, it is recommended to heat the sample to no more than 37° for 5 min in the SDS loading buffer prior to analysis.[12] Including chelating agents, such as EDTA (100 m*M*) in the elution buffer, can facilitate maximal elution of proteins from the resin. This treatment will strip the metal atoms away from the matrix, resulting in contamination of the elute. The presence of the chelating agent or the metal in the eluate may interfere with enzyme activity. Moreover, the matrix cannot be reused following the use of a chelating agent without recharging.

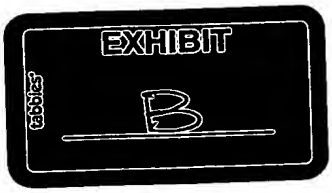
Protocol: Native Purification of a Soluble Polyhistidine-Tagged Protein

The following protocol is one that is designed for the purification of the soluble ERK2 protein tagged with six N-terminal histidine residues from *E. coli* utilizing a Ni^{2+}–NTA resin under nondenaturing conditions (see Fig. 2).[24] While this protocol may need minor optimization for other

[24] D. J. Robbins, E. Zhen, H. Owaki, C. A. Vanderbilt, D. Ebert, T. D. Geppert, and M. H. Cobb, *J. Biol. Chem.* **268**, 5097 (1993).

APPENDIX 2

September 2003



EasyXpress™ Protein Synthesis Handbook

EasyXpress Protein Synthesis Mini Kit
EasyXpress Protein Synthesis Maxi Kit
EasyXpress Linear Template Kit

For in vitro synthesis of recombinant proteins and generation of linear expression templates by PCR

QIAGEN

Contents

Kit Contents

EasyXpress Protein Synthesis Kit	**Mini Kit: For 20 x 50 µl reactions**	**Maxi Kit: For 4 x 1 ml reactions**
Cat. no.	**32502**	**32506**
EasyXpress *E. coli* Extract (colorless snap-cap)	20 x 17.5 µl	4 x 350 µl
EasyXpress Reaction Buffer (blue screw-cap)	1 x 400 µl	4 x 450 µl
RNase-free H_2O (colorless screw-cap)	1 x 1.9 ml	1 x 1.9 ml
EasyXpress Positive-Control DNA (yellow screw-cap)	1 x 50 µl	4 x 50 µl
EasyXpress Linear Template Kit (20)		**For 20 two-step PCRs**
Cat. no.		**32703**
ProofStart™ DNA Polymerase (orange screw-cap)		40 µl (100 U)
10x ProofStart PCR Buffer (blue screw-cap)		1 ml
25 mM $MgSO_4$ (yellow screw-cap)		1 ml
dNTP Mix (10 mM each) (purple screw-cap)		200 µl
5x Q-Solution (green screw-cap)		400 µl
RNase-free H_2O (colorless screw-cap)		1.9 ml
EasyXpress Positive-Control DNA (PCR) (white screw-cap)		50 µl (20 control reactions)
Positive-Control Sense Primer (white screw-cap)		15 µl
Positive-Control Antisense Primer (white screw-cap)		15 µl
Strep N-term. Sense Primer (yellow screw-cap)		40 µl
Strep C-term. Antisense Primer (brown screw-cap)		40 µl
6xHis N-term. Sense Primer (yellow screw-cap)		40 µl
6xHis C-term. Antisense Primer (brown screw-cap)		40 µl
No tag N-term. Sense Primer (yellow screw-cap)		40 µl
No tag C-term. Antisense Primer (brown screw-cap)		40 µl
XE-Solution (green screw-cap)		40 µl

Protocol: Purification of 6xHis-tagged Proteins Using Ni-NTA Magnetic Agarose Beads

This protocol can be used to efficiently purify 6xHis-tagged proteins from 50 µl EasyXpress in vitro translation reactions.

Materials to be supplied by user

- In vitro translation reaction containing 6xHis-tagged protein
- Ni-NTA Magnetic Agarose Beads (QIAGEN cat. no. 36111)
- Magnetic separator (e.g., 12 tube-magnet, QIAGEN cat. no. 36912)
- Ni-NTA Beads Binding Buffer
- Ni-NTA Beads Wash Buffer
- Ni-NTA Beads Elution Buffer

Buffer compositions are provided in the Appendix on page 50.

Procedure

1. **Resuspend Ni-NTA Magnetic Agarose Beads by vortexing for 2 s and then immediately pipet 150 µl of the 5% Ni-NTA Magnetic Agarose Bead suspension into a 1.5 ml reaction tube.**

   Note: Care is necessary to ensure that constant amounts of beads are pipetted. The beads will settle if the suspension is not agitated regularly. 100 µl magnetic-bead suspension has a binding capacity of 30 µg 6xHis-tagged DHFR (24 kDa). If significantly different amounts of tagged protein are present in your lysate, the volume of magnetic-bead suspension should be varied accordingly. However, use of volumes less than 10 µl are not recommended due to the associated handling problems — smaller volumes are difficult to pipet and may lead to uneven distribution of beads and reduced reproducibility.

2. **Place the reaction tube on a magnetic separator for 1 min. Carefully remove supernatant with a pipet.**
3. **Remove the tube from the magnetic separator and add 500 µl Ni-NTA Beads Binding Buffer. Briefly vortex, place the reaction tube on a magnetic separator for 1 min, and remove supernatant.**
4. **Pipet 700 µl Ni-NTA Beads Binding Buffer into the tube containing the Ni-NTA Magnetic Agarose Beads and mix by pipetting up and down.**
5. **Pipet the 50 µl in vitro translation reaction into the tube containing the Ni-NTA Magnetic Agarose Beads suspension.**
6. **Mix the suspension gently on an end-over-end shaker for 60 min at 4°C.**

7. **Place the tube on a magnetic separator for 1 min and remove supernatant with a pipet.**

   Tubes may be briefly centrifuged before placing on the magnetic separator, to collect droplets of suspension from the tube caps.

8. **Remove tube from the magnet, add 500 µl of Ni-NTA Beads Wash Buffer, mix the suspension, place the tube on a magnetic separator for 1 min, and remove buffer.**

9. **Repeat step 8 one or two times.**

   Buffer remaining after the final wash should be removed completely.

10. **Add 50 µl of Ni-NTA Beads Elution Buffer to the beads, mix the suspension, incubate the tube for 1 min, place for 1 min on magnetic separator, and collect the eluate.**

    Tubes may be centrifuged before placing on the magnetic separator, to collect droplets of suspension from the tube caps.

11. **Repeat step 10.**

    Most of the 6xHis-tagged protein will elute in the first elution step.

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